Collagen affinity coating for surface binding of decorin and other biomolecules: Surface characterization

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(Received 13 April 2017; accepted 12 June 2017; published 28 June 2017)

The development of biomaterials that promote tissue reconstruction and regeneration can reduce the low level, chronic inflammation and encapsulation that impact the performance of today's medical devices. Specifically, in the case of implantable sensors, the host response often leads to poor device performance that discourages permanent implantation. Our goal is to present on medical implants bioactive molecules that can promote healing rather than scarring. Localized delivery of these molecules would also minimize the possibility of adverse tissue reactions elsewhere in the body. Toward this end, the authors have developed a collagen affinity coating that binds a number of potential healing molecules and can be attached to the surface of an implanted biomaterial. This allows the creation of a wide variety of natural surface coatings that can be evaluated and tailored to promote the desired healing response. To demonstrate the efficacy of this collagen affinity coating to biospecifically bind promising healing molecules to type I collagen *in vivo*, the antifibrotic proteoglycan decorin was utilized. Decorin binds and renders ineffective the protein transforming growth factor beta (TGFβ) that induces collagen scar production. Thus, an assembled, supramolecular structure of biomaterial-collagen-decorin-TGFβ is formed. A decorin surface coating was created and characterized, illustrating the potential of this type I collagen affinity coating for widespread use with a variety of promising healing molecules. Future studies will test the implant efficacy of this type of coating. © *2017 American Vacuum Society*. [http://dx.doi.org/10.1116/1.4989835]

I. INTRODUCTION

Collagen is the most abundant protein in the human body and its diverse functions include providing mechanical integrity and signaling by serving as a depot for cytokines and growth factors. In earlier studies, we have exploited the ability of type I collagen to biospecifically bind many proteins by synthesizing an affinity coating to present the matricellular protein osteopontin on surfaces in a biologically active form.[1] Other studies have also taken advantage of biomolecule binding to collagen to create unique surfaces and interphases.[2–4] At least 50 biological molecules have been identified, which also interact with type I collagen,[5] and approximately half of these have their specific sites of interaction pinpointed on the collagen fibril. The most active region for sites of interaction on the fibril is the C-terminus portion.[5] It is probable that molecules noncovalently attached to collagen I are in a conformation appropriate for biointeraction. The type I collagen affinity coating described here thus holds the potential to noncovalently attach a variety of biological molecules to any surface onto which it is immobilized. By attaching specific molecules of interest and evaluating the healing responses to these molecules, we can tailor the healing response around an implant.

Among the numerous molecules that have been shown to biospecifically interact with type I collagen is the proteoglycan decorin. This 70 kD molecule is a member of the family of small leucine-rich proteoglycans.[6] Decorin has been shown to bind near the C-terminus of type I collagen[7] with an apparent dissociation constant of 10^{-8} M.[8] The core protein consists of 12 leucine rich repeats (LRRs) and contains a single chondroitin or dermatan sulfate chain near the N terminus. The binding site for collagen on the decorin molecule is located between LRRs 5 and 6.[9,10] Decorin is essential in collagen organization during fibrillogenesis as demonstrated by a decorin knockout mouse with fragile connective tissues and irregular collagen fibril thicknesses and organization.[11] In healthy connective tissues, decorin is found near the C-terminus of each fibril with the glycosaminoglycan side chain forming a bridge between decorin molecules along adjacent fibrils.

In numerous fibrotic disease models, decorin has been shown to reduce fibrosis and improve outcomes.[12–15] The antifibrotic effects of decorin have been attributed to its ability to bind and inactivate transforming growth factor.[16,17] This interaction has been shown to reduce fibrosis in a rat model of kidney fibrosis,[12,13] a hamster model of lung fibrosis,[14] and a rat arterial injury model.[15] Decorin has also been shown to reduce smooth muscle cell migration, proliferation, and collagen synthesis due to platelet derived growth factor stimulation after arterial injury.[18] Decorin injection into a mouse laceration model resulted in reduced scarring and improved muscle recovery.[19,20]

In this study, we demonstrate and characterize a novel biomaterial surface coating formed by the attachment of decorin to a collagen affinity coating. Future studies will discuss the biological response to these materials. The

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successful attachment of decorin to the surface of the collagen affinity coating is further proof for the widespread applicability of this coating to attach a number of potentially interesting, bioactive molecules. The ultimate goal will be tailoring the biological response to implants to one of the tissue reconstruction rather than scarring and encapsulation.

II. MATERIALS AND METHODS

A. Casting pHEMA gels

Poly-2-hydroxyethyl methacrylate (pHEMA) gels were synthesized by mixing 5 g of the ophthalmologic grade 2-hydroxyethyl methacrylate (HEMA) monomer (Polysciences, Inc, Warrington, PA) with 0.2 g of tetraethylene glycol dimethacrylate (Polysciences, Inc), 1.5 g of ethylene glycol and 1 g of water, followed by the addition of the initiators, 0.5 g of sodium metabisulfite (15% w/w in water) and 0.5 g ammonium persulfate (40% w/w in water). Initiator salts were obtained from Sigma-Aldrich, Inc. The solution was polymerized at room temperature between two glass plates separated by 1 mm thick Teflon spacers. The hydrogel was soaked in deionized water to facilitate the removal of glass plates and spacers and remove the unreacted monomer. The pHEMA sheet remained in deionized water for several days with fresh water changes each day. Disks (5 mm diameter) were cut using a biopsy punch and dried in a vacuum desiccator for 3 days. Dry disks were stored in glass scintillation vials, backfilled with argon, and sealed with Parafilm.

B. Spincoating pHEMA

Spincoated pHEMA disks were prepared for use in ultra-high-vacuum surface analysis techniques including electron spectroscopy for chemical analysis (ESCA) and time-of-flight secondary ion mass spectrometry (ToF-SIMS). Glass coverslips (12 mm diameter) were coated with a thin layer of pHEMA (Scientific Polymer Products, Inc., Ontario, NY) as previously described.[1] The glass coverslips were first cleaned by sonication in a series of solvents, dried, and coated on both sides by pipetting 10–15 microliters of 2.5% (w/v) ethyl methacrylate-3-(trimethoxysilyl)propyl methacrylate copolymer (synthesized in house) in ethyl acetate onto the surface while spinning at 4000 rpm for 20 s. The coated disks were placed in an oven at 60 °C overnight. This was followed by 2–1 h rinsing with ethyl acetate under stirring and a subsequent 10 min methanol rinsing. The spincoating procedure was repeated with 15–20 μl of 2.5% (w/v) pHEMA in methanol until all disks were coated on both sides with pHEMA.

C. Collagen immobilization

Hydroxyl groups on pHEMA surfaces were activated with carbonyldiimidazole (CDI) (Aldrich Chemical Company, Milwaukee, WI) allowing covalent linkage to primary amine groups found on type I collagen. Dry pHEMA disks underwent three 1,4-dioxane rinsing steps followed by reaction with 20 mM CDI in dioxane for 2.5 h at 50 °C. The CDI solution was removed, and each sample was rinsed three times with dioxane to remove any unreacted CDI. A large excess of type I collagen from rat tail tendon (concentration 300 μg/ml) (BD Biosciences) in sodium carbonate–bicarbonate buffer (*p*H 10.2) was added. The surfaces were allowed to bind overnight at 4 °C. Surfaces were then rinsed three times with sodium carbonate–bicarbonate buffer.

D. Preparation of decorin coating

Solutions of 30, 50, 100, and 150 μg/ml of decorin (bovine articular cartilage, Sigma-Aldrich) in phosphate buffered saline (PBS, *p*H 7.4) were prepared. Collagen coated samples were allowed to naturally bind to decorin in the solution (0.33 ml/sample) overnight at 4 °C. Samples were then rinsed three times with PBS. A schematic of the multistep coating is presented in Fig. 1.

E. Fluorine derivatization reaction

In order to confirm successful CDI activation of our surfaces, a fluorine derivatization reaction was employed. The

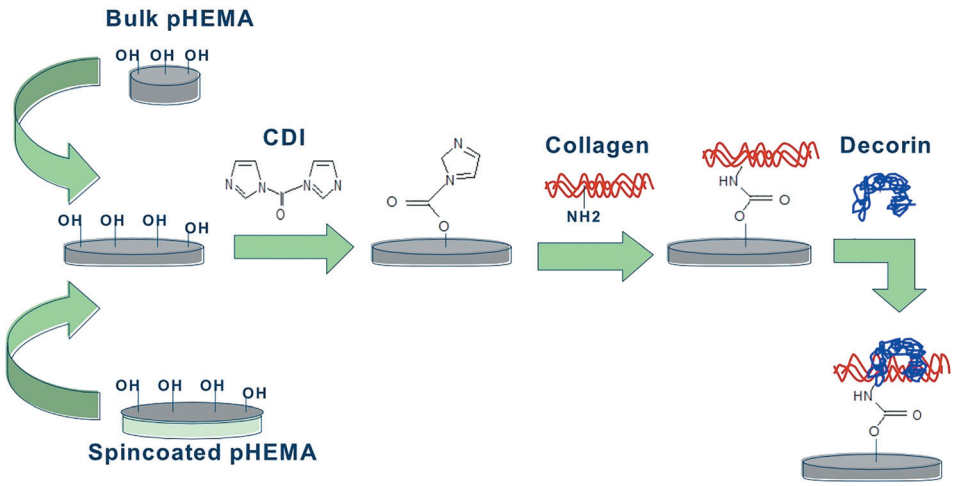

FIG. 1. Schematic of coating preparation. Hydroxyl groups on the pHEMA surface are activated by CDI. Collagen is covalently bound followed by the natural attachment of decorin.

fluorinated molecule 2,2,2-trifluoroethylamine (TFEA) (Aldrich) contains a primary amine that can react with the imidazole carbamate from CDI. Spincoated pHEMA disks, disks activated with CDI only, and disks activated with CDI plus the collagen affinity coating reacted with 1.5 ml of TFEA overnight at room temperature. The samples were placed onto a microscope slide, and the slide was placed in a large glass test tube. TFEA was injected into the tube in the space underneath the microscope slide. The tube was backfilled with nitrogen, sealed with a Teflon-coated stopper, and left overnight to react. Samples were placed into a desiccator to remove unreacted TFEA and then analyzed by ESCA to determine the concentration of fluorine in the surface zone.

F. Electron spectroscopy for chemical analysis

To measure fluorine concentrations from fluorine-derivatized surfaces as well as to verify the successful addition of protein to our surfaces, ESCA was utilized. Surface compositions for top 50–80 Å of samples were analyzed on a Surface Science Instruments X-probe ESCA instrument using an aluminum $K_{\alpha 1,2}$ monochromatized x-ray source. Spincoated pHEMA samples were pumped down to reach a pressure of approximately 10^{-9} Torr. An electron flood gun was used to reduce surface charging. A hemispherical energy analyzer was used to measure the energy of emitted electrons. The samples were analyzed at a 55° angle with respect to the surface normal. Analysis of spectra was conducted using Surface Science Instruments analysis software with the hydrocarbon peak in the high resolution carbon spectrum set to 285.0 eV as a reference for the binding energy scale.

G. Time-of-flight secondary ion mass spectrometry

To obtain detailed molecular information from outermost 10–20 Å of protein-coated surfaces, samples were evaluated using ToF-SIMS. Spincoated pHEMA samples with the CDI-immobilized collagen affinity coating were evaluated. The decorin surface coating onto the CDI-immobilized collagen affinity coating from the 30 μg/ml solution concentration was evaluated. As an additional control, a CDI-immobilized decorin coating (i.e., decorin without collagen) was created by the same method described previously for CDI-immobilized collagen using a decorin solution concentration of 30 μg/ml.

Static SIMS positive ion spectra were obtained using a Physical Electronics PHI Model 7200 reflectron instrument (Eden Prairie, MN) with an 8 keV Cs^+ ion source. The primary ion dose was maintained below 1×10^{13} ions/cm^2 over an analysis area of 100×100 μm. All spectra were calibrated to CH_3^+, $C_2H_3^+$, $C_3H_5^+$, and $C_7H_7^+$ peaks with mass calibration errors less than 5 ppm before subsequent analysis. Peaks specific to fragments from any of the 20 amino acids as identified based on the work by Lhoest *et al.*[21] were selected for further analysis.

Selected amino acid peaks were converted into a peak table and then analyzed using the multivariate pattern recognition technique, principal component analysis (PCA). PCA was performed as previously described[22] using algorithms programmed into MATLAB (v 5.0, The Math Works, Inc., Natick, MA). Each spectrum was mean centered and normalized to the total intensity of the spectrum before analyzing.

H. Quantification of surface-bound decorin

The amount of decorin on the surface of pHEMA specimens was determined using ^{125}I radiolabeling. Decorin of 100 μg was labeled using Iodobeads (Pierce Chemical Co. Inc) according to manufacturer's instructions. Two washed and dried beads were added to 0.25 ml of PBS and 1 mCi of ^{125}I and reacted for 5 min. Decorin of 100 μg in 0.5 ml of PBS was added to the iodine solution and reacted for 15 min. Two Biorad Econo-Pac 10 DG chromatography columns were drained and rinsed twice with PBS. The labeled protein solution was added to the first column. PBS (0.5 ml) was added to the column, and fractions were collected from the base. This was repeated until 40 0.5 ml fractions were collected for both the labeled protein and unbound iodine. Radioactivity of all 40 fractions was measured, and the three fractions containing the labeled protein were added to the second column. Fraction collection was repeated, and the three fractions containing the highest radioactivity were retained. The protein concentration of the combined three fractions containing the highest radioactivity could be measured by UV spectrometry, and from that concentration, radioactivity (counts) per microgram of protein could be counted.

Solutions of 30, 50, 100, and 150 μg/ml of decorin were prepared and spiked with iodinated decorin to yield sufficiently "hot" protein so that adsorbed levels permitted statistically reliable counting. Iodinated decorin was counted as "buffer" for decorin concentration calculations. Bulk pHEMA disks of 5 mm diameter were used for all radiolabeling studies. pHEMA disks without labeled protein, pHEMA with adsorbed decorin, and pHEMA with adsorbed collagen followed by decorin were prepared in addition to the covalently attached collagen affinity coating with increasing concentrations of decorin in the solution. Decorin attachment and collagen affinity coatings were prepared as described previously, and activity was measured in a gamma counter (1 min counts). After initial counts, sample tubes were incubated at 37 °C and the amount of decorin remaining on surfaces over time was measured. At each time point, solutions were removed from tubes and replaced with fresh PBS buffer and activities were measured.

III. RESULTS AND DISCUSSION

A. Fluorine derivatization to confirm CDI activation

A fluorine derivatization reaction was used to ensure the successful activation of pHEMA surfaces as well as evaluate the presence of reactive groups present after covalent immobilization of collagen. ESCA survey scans showed undetectable levels of fluorine on unmodified pHEMA surfaces, 8.6 at. % fluorine on CDI-activated surfaces and 2.8 at. % fluorine on collagen affinity coatings. The theoretical fluorine

composition is 17.6 at. % for activation of every hydroxyl group on the surface and 100% binding of TFEA. Assuming that steric hindrance of CDI in binding to free hydroxyl groups is the cause of lower actual fluorine composition, we can estimate that approximately 50% of the available hydroxyl groups are activated by CDI. High resolution carbon scans (Fig. 2) demonstrate the appearance of a peak consistent with C-F_3 at a chemical shift of 7.7 eV from the hydrocarbon peak on the CDI-activated surface. The intensity of this C-F_3 peak is reduced on the collagen affinity coating surface.

B. Verification of the surface composition by ESCA

ESCA was used to characterize the composition of CDI-activated and protein-coated surfaces. Survey scans provided a carbon/oxygen ratio of 67.3/32.7 for spincoated pHEMA surfaces, close to theoretical values of 66.7/33.3. CDI-activation of the surface introduces a nitrogen peak to the composition scan. For 1 to 1 binding of CDI to free hydroxyl groups on pHEMA, a theoretical %N of 12.5 would be obtained. Calculations based on the measured nitrogen signal of 5.7% result in an activation of 45.6% of hydroxyl groups present. This is in agreement with the CDI/OH ratio obtained from fluorine derivatization. An increase in the nitrogen composition to 10.1% indicated the addition of collagen to the CDI-activated surface. The subsequent addition of decorin resulted in similar levels of nitrogen. Survey scans from CDI-activated spectra are provided in Fig. 3.

C. Analysis of immobilized protein surfaces by ToF-SIMS

Analysis of SIMS data was focused on elucidating differences in protein structures and compositions among decorin (DCN), collagen (COL), and collagen + decorin (COL+DCN) surfaces. Peaks specific to the 20 amino acids were selected and used to determine differences between these protein-coated surfaces through PCA. The first two principal components (PCs) contained 98% of the intensity differences between surfaces among the selected peak set. The plot of PC1 versus PC2 in Fig. 4 shows distinct clustering of the three separate surfaces with the strongest separation along PC2. This clustering indicates differences in the amino acid compositions of the three surfaces. The loading plot of PC2 (Fig. 5) provides information on which specific amino acids are primarily contributing to this separation. The most pronounced differences can be attributed to a strong proline peak (m/z = 70) in COL samples, whereas DCN samples contain strong leucine (m/z = 86) and lysine (m/z = 84) peaks. The large proline content of collagen and the characteristic leucine rich repeats in the structure of decorin lend

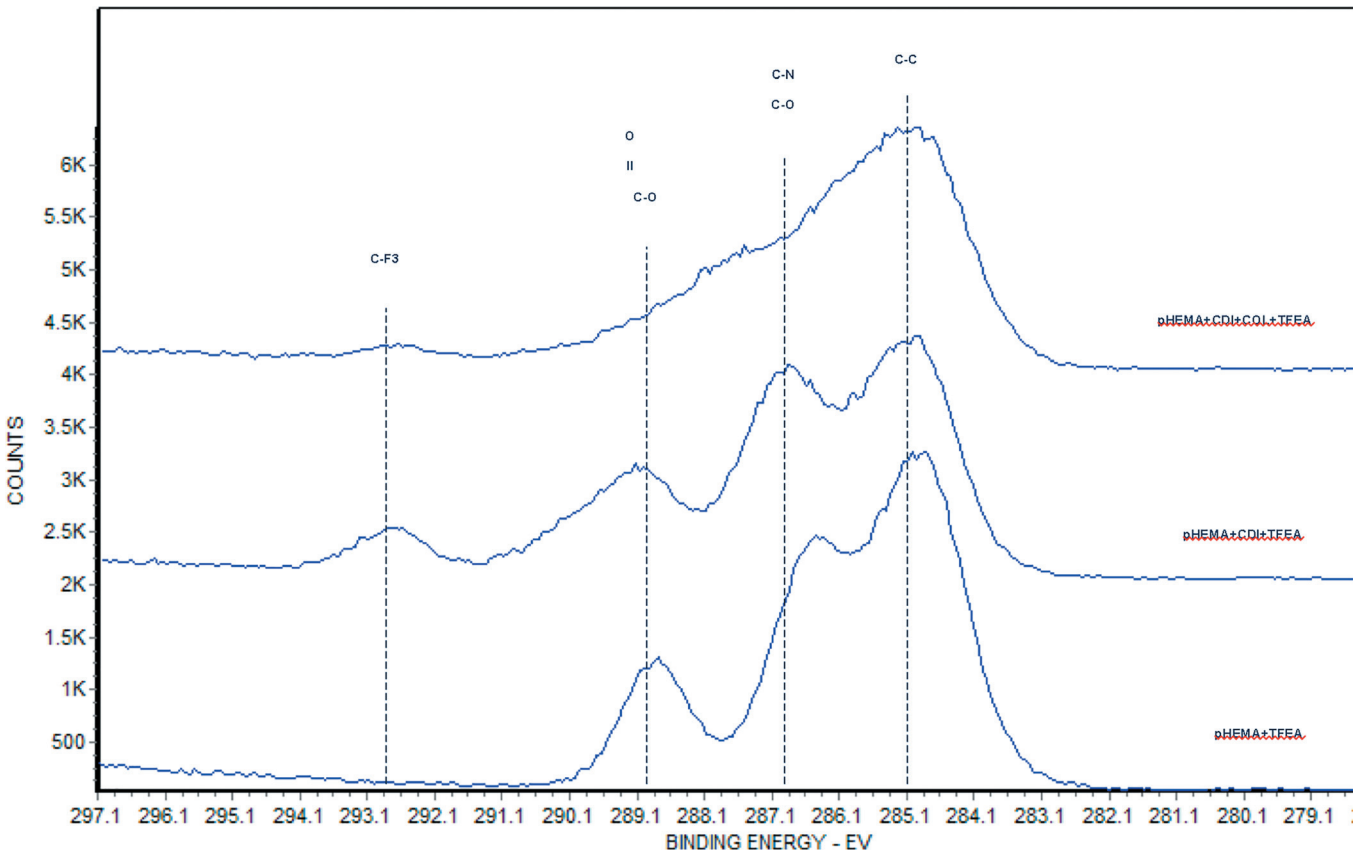

FIG. 2. High resolution C1s scans of TFEA derivatized surfaces showing no C-F_3 peak on the pHEMA surface (bottom), while the CDI activated surface (middle) demonstrates the appearance of a C-F_3 peak at a shift of 7.7 eV from the C-H peak at 285.0. A reduced C-F_3 peak is present on the TFEA reacted CDI+collagen surface (top).

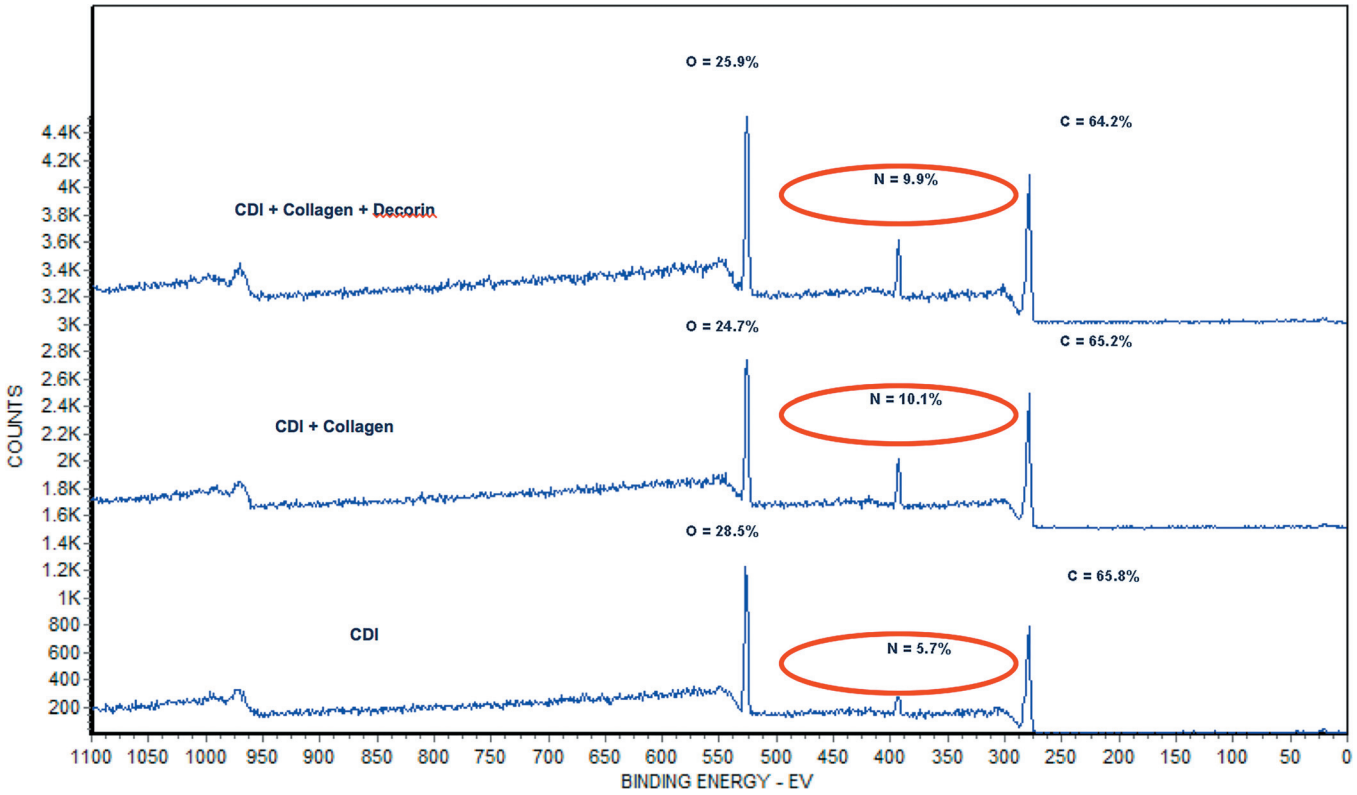

FIG. 3. ESCA composition scans demonstrating the presence of a N peak on the CDI-activated pHEMA surface (bottom) with an increase in %N for the collagen-immobilized surface (middle) and the collagen immobilized surface with bound decorin (top).

support to these findings in SIMS spectra. The separation of COL+DCN from DCN surfaces indicates that we are likely seeing both proteins at our coating surface and we do not have complete coverage of collagen by decorin.

D. Quantification of decorin by radiolabeling

The amount of decorin able to bind to the collagen affinity coating was measured via radioiodination of decorin. Decorin solution concentrations from 30 to 150 μg/ml were used to evaluate binding. Binding was found to be dose-dependent. Quantities of decorin bound to each surface can be seen in Fig. 6. At the highest solution concentration evaluated, 150 μg/ml, the quantity of decorin bound to the surface was 300 ng/cm^2, which is consistent with a monolayer of decorin at the surface. However, we did not investigate higher solution concentrations, and so, we do not know if the surface is saturated. Radiolabeling studies confirmed successful binding of decorin to collagen affinity coatings in quantities that are reasonable for a monolayer of protein. Amounts of decorin remaining on surfaces over time at 37 °C in the PBS buffer solution are given in Fig. 7. The majority of decorin was released into the solution in the first 24 h of incubation, indicating the reversible attachment of decorin to our collagen affinity coating. These results also demonstrate the ability to control surface quantities of decorin by controlling the solution concentration for decorin binding.

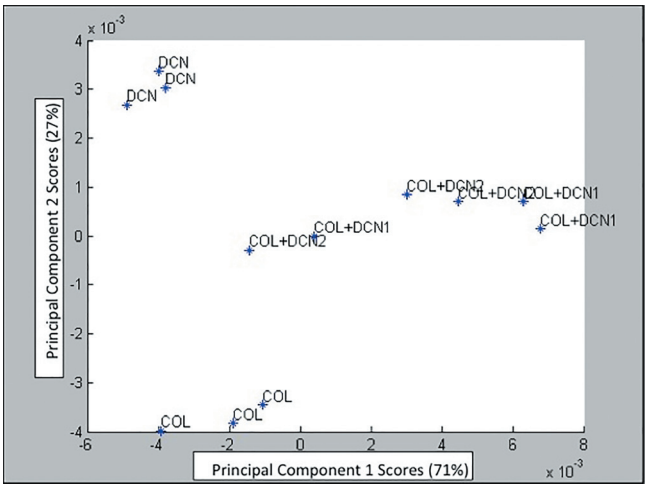

FIG. 4. Plot of PC1 vs PC2 showing the separation of decorin (DCN), collagen (COL), and collagen + decorin (COL+DCN) surfaces.

E. Discussion

We have demonstrated the successful and reversible attachment of the extracellular matrix proteoglycan decorin to the surface of our collagen affinity coating. Attachment is in a dose-dependent manner. We have previously demonstrated the successful attachment of the matricellular protein osteopontin to our collagen affinity coating,[1] and thus, the successful binding of decorin further proves the applicability of this coating for widespread use in attaching a number of biologically active molecules.

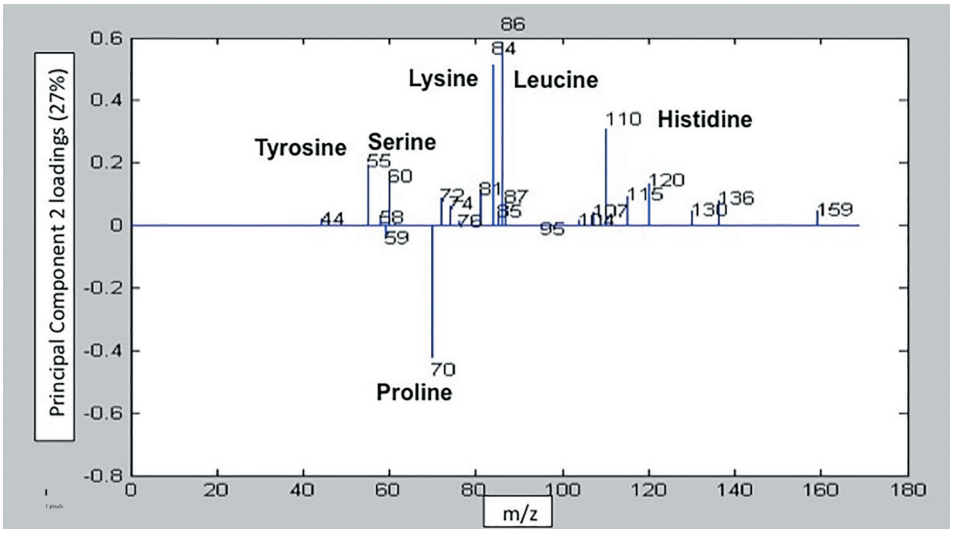

FIG. 5. Loading plot of PC2 demonstrating specific amino acid peaks which contribute to the separation of decorin, collagen, and collagen + decorin surfaces.

Most of the decorin is only weakly complexed with the surface collagen (Fig. 7). Upon implantation, a fully loaded decorin surface will release free decorin at the site of implantation. This free decorin should act to inhibit early events, leading to scar formation similar to that noted by Fukushima *et al.*[19] and Sato *et al.*[20] However, at least 30 ng/cm^2 of decorin remain tightly bound to the surface. This surface-resident decorin could act to inhibit the scarlike foreign body capsule at longer times.

At least 50 naturally occurring proteins, proteoglycans, growth factors, and other biological molecules are known to bind type I collagen.[5] The wide variety of molecules that can bind to type I collagen suggests possibilities for creating multifunctional surface coatings. Several different molecules could be attached to the same affinity coating, each of which would contribute to the overall desired synergistic effect. As illustrated with decorin binding, the surface concentration of each molecule of interest could be controlled by selecting an appropriate solution concentration for loading. Thus, specific molecules could be selected based on the desired effect for the overall coating, each with a distinct binding region on the collagen fibril, and attached at a controllable surface quantity.

With current technology allowing creation of synthesized peptides and protein mimics, the pool of candidate molecules for surface attachment to collagen expands dramatically. Collagen binding domains could be added to any number of synthetic molecules to modify surface properties

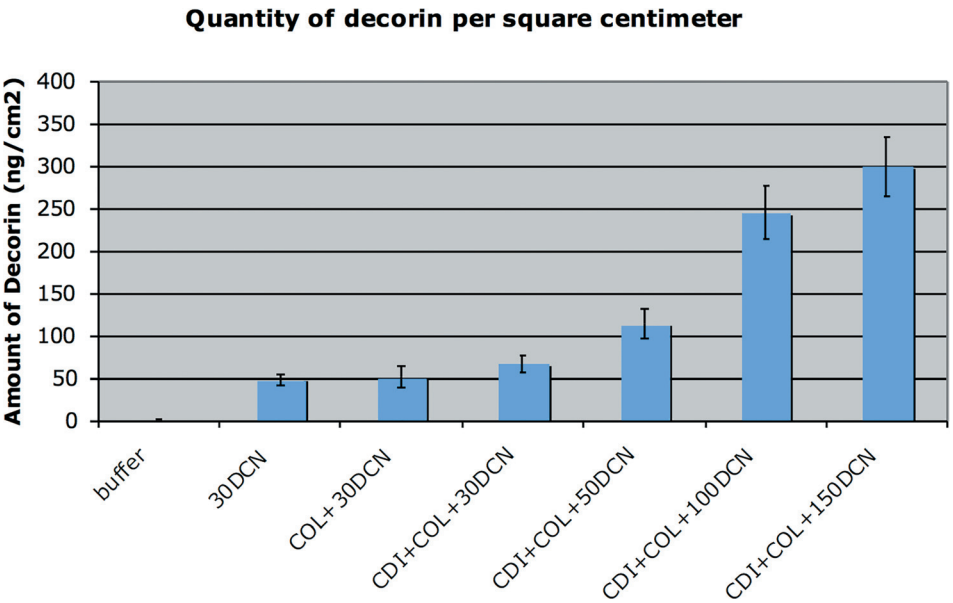

FIG. 6. Quantity of decorin in ng/cm^2 present on the surface of pHEMA disks. Treatments include pHEMA disks with no protein (buffer), adsorbed decorin (30DCN), adsorbed collagen and decorin (COL+30DCN), and CDI-activated pHEMA with immobilized collagen with increasing concentrations of decorin in μg/ml (CDI+COL+DCN).

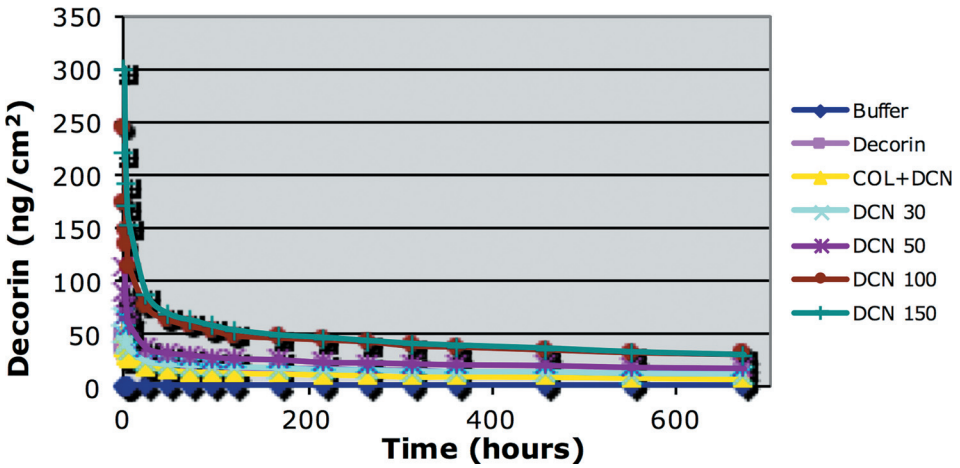

FIG. 7. Quantity of decorin retained on surfaces over time at 37 °C. Treatments include pHEMA disks with no protein (buffer), adsorbed decorin (30DCN), adsorbed collagen and decorin (COL+DCN), and pHEMA with CDI-immobilized collagen and increasing concentrations of decorin in μg/ml (DCN 30, DCN 50, DCN 100, and DCN 150).

or elicit specific biological responses. Peptidoglycans with synthetic collagen binding domains attached to glycosaminoglycan side chains were strategically designed to mimic the class of small leucine rich proteoglycans in which decorin is a member.[23,24] One peptidoglycan in particular contains a collagen binding domain from the core protein of decorin, SYIRIADTNITGC (Dc13), attached to a dermatan sulfate side chain.[10,24] Another peptidoglycan took its collagen binding domain from the platelet receptor to type I collagen, RRANAALKAGELYKSILYGC (SILY).[24,25] Both these peptidoglycans have demonstrated the ability to bind type I collagen and modulate collagen fibrillogenesis. These peptidoglycans and other biologically active synthetic molecules would be ideal candidates for use in collagen affinity coatings to develop multifunctional surface coatings that might promote healing and integration.

The applicability of the collagen affinity coating is not limited to pHEMA or even hydrogel materials. Most surfaces are suitable for HEMA monomer plasma deposition. Under appropriate conditions, such plasma depositions can be delamination-resistant and can contain high levels of –OH groups that can be postreacted for immobilization.[26] Additionally, surfaces with amine groups are also potential candidates. This collagen affinity coating can be applied to a wide variety of medical products and devices and allows for the creation of numerous bioactive coatings.

Future studies include the *in vivo* assessment of the surfaces described here and the creation of tailor-made multifunctional surfaces by exploiting the wide variety of molecules that bound to collagen. We can strategically select candidates with desirable biological activities, which bind to distinct regions along the collagen fibril.

IV. SUMMARY AND CONCLUSIONS

This work has demonstrated the successful binding of decorin to the surface of an implant via a type I collagen affinity coating. Each step of the coating process has been characterized and verified, including the CDI activation of hydroxyl groups on the pHEMA surface, covalent immobilization of type I collagen, and natural binding of decorin. Fluorine derivatization demonstrated an activation efficiency of approximately 50% of surface hydroxyl groups by CDI. The quantity of decorin on the surface was approximately 300 ng/cm^2 based on radiolabeling studies, which is consistent with a monolayer of the proteoglycan based on the dimensions of the decorin molecule. The successful binding of decorin to the collagen affinity coating further proves the applicability of this coating for widespread use in attaching a number of biologically active molecules. Future work will include evaluating the healing response of this decorin coating *in vivo* as well as exploring binding and the healing potential of numerous additional molecules, which interact with collagen naturally. This collagen affinity coating provides the ability to attach several molecules in various quantities and may allow us to tailor the host response to promote healing.

ACKNOWLEDGMENTS

The authors gratefully acknowledge the University of Washington Engineered Biomaterials Engineering Research Center, originally funded by NSF EEC-9529161, and the Optical Microscopy and Image Analysis Shared Resource, funded by NSF Grant No. EEC-9872882. The National ESCA and Surface Analysis Facility for Biomedical Problems (NESAC/BIO), funded by the NIH-NIBIB, provided support and instrumentation for surface analysis studies. Additional funding was received from an NSF Graduate Research Fellowship. Discussions with Thomas N. Wight are also appreciated.

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